

Review

Y2K+1 state-of-the-art on non-peptide phosphoantigens, a novel category of immunostimulatory molecules

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ABSTRACT – Some human T cells are activated *in vivo* and *in vitro* by small non-peptide antigens, so-called phosphoantigens. Since their discovery in 1994, several reports have continuously documented novel members of this category of immunostimulatory molecules. This article reviews the current knowledge on their biochemical properties. © 2001 Éditions scientifiques et médicales Elsevier SAS

pyrophosphate / antigens / gamma-delta lymphocytes / activation

1. Definition and chronological background

Phosphoantigens are small-molecular-weight molecules with phosphorylated structures that selectively activate some human T cells expressing $\gamma\delta$ T-cell receptors (TCRs). To review the types and properties of phosphoantigens and the few functionally related compounds known so far, we will first summarise some general information useful to the reader regarding the read-outs of phosphoantigen bioactivity on the reactive lymphocytes. For a recent review about phosphoantigen-mediated activation of G9D2 cells in physiopathological contexts see Microbes and Infection's recent special issue on $\gamma\delta$ T cells [1] and more specifically [2, 3].

1.1. Human $\gamma\delta$ cells: nature and activities

Since their discovery in the 1980s $\gamma\delta$ T cells have continuously constituted a puzzling issue for immunologists. $\gamma\delta$ cells are present in most vertebrates and located in blood and in lymphoid and in non-lymphoid tissues such as skin, epithelia and mucosa. Although they differentiate in the thymus [4] but also in other sites such as the intestine or foetal liver, they are nevertheless authentic (CD3⁺) T lymphocytes, as they use TCR encoded by γ and δ loci to recognise antigens. In humans, these T lymphocytes most frequently have CD4⁺CD8⁺ cell surface pheno-

types [5, 6] and also display receptors usually characterising NK cells (NKR) [7–11]. They have a high frequency in circulating blood of healthy adults: usually 1–10% of total lymphocytes. Furthermore, the expression of a restricted set of TCR variable genes (TCR V γ 9V82 referred to as G9D2) in the vast majority (75–90% of blood $\gamma\delta$ cells [12–14]) make the single G9D2 cell subset the most prominent one in the adult peripheral repertoire [4]. G9D2 T cells are cytotoxic lymphocytes [15]: they kill target cells by perforine/granzyme, FAS-L- and TNF- α -mediated pathways and secrete antimicrobial granulysin (F. Dieli, personal communication).

The G9D2 subset originates from thymic precursors and expands in peripheral blood by antigen-driven selection. Since presenting elements of MHC or MHC-related molecules do not seem to be required for G9D2 T-cell activation by specific ligands, whether positive and negative selection in the thymus or in the periphery is required still remains unknown. However MHC class I molecules are known to directly contribute to the G9D2 activation threshold: the vast majority of mature, peripheral G9D2 cells express NKR such as, most notably, CD94/NKG-2, which mediate intracellular inhibitory signalling upon surface interaction with their nominal MHC ligand [16].

G9D2 T cells are viewed as effectors at the border of innate and adaptive immunity. These cells express a TCR resulting from recombination of germline DNA segments and acquire a specificity (cf. *infra*) typifying adaptive immunity and yet, this is a broad specificity, with some degeneracy conferring the capability to recognise a diverse set of pathogens. The G9D2 response to Ag recognition is

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mounted very quickly, with neither the need of antigen-presenting cells nor localisation in a secondary lymphoid organ. So, in this respect, G9D2 T cells behave like innate immunity cell effectors.

The physiological functions fulfilled by G9D2 cells in immunity are not the focus of this review, but it remains worth mentioning that besides cytotoxicity, these cells respond to antigenic activation by Th-1 cytokine [17–19] and chemokine release [20], IL-2-dependent proliferation, induced apoptosis (AICD, [21–26]) memory [27–29] and release of NO [30].

In humans, G9D2 are known to be involved in pathology involving intracellular pathogens like *Mycobacteria*, *Plasmodium* or *Toxoplasma* (cf. infra).

1.2. Activation by non-peptide antigens

The seminal basis on the activation of human G9D2 cells by natural antigens was the finding of a selective recognition of ligands present in *Mycobacterium tuberculosis* extracts which surprisingly cross-reacted with the cell surface of Daudi Burkitt's lymphoma cells [31]. Although indirect evidence suggested that hsp65 could be the responsible cross-reactive antigen conserved from microorganisms to human cells [31, 32], further careful dissection of the antigenic material present in mycobacterial extracts evidenced its difference from hsp65 [33]. The first demonstration of its non-peptidic nature came from K. Pfeffer's studies, which definitively showed that the G9D2 stimuli present in *M. tuberculosis* compounds were protease-resistant low-molecular-weight (1–3 kDa) compounds [34, 35] that could bind to plant lectins and actually turned out to differ from Daudi's tumoral antigens [36].

2. Natural phosphoantigens

2.1. Mycobacterial ligands

When attempting to isolate the antigen for $\gamma\delta$ cells present in *M. tuberculosis* cultures, P. Constant et al. [37] separated four such molecules, with different non-peptidic structures. Two of the smallest antigens (TUBag1 and TUBag2) were alkaline phosphatase-sensitive pyrophosphate monoesters of an unidentified residue (X moiety), while the largest molecules were alkaline phosphatase-resistant nucleotide conjugates containing thymidine 5'-triphosphate γ -diester (TUBag4, [37]) and uridine 5'-triphosphate γ -diester (TUBag3, [38]). Nucleotide ligands are present in cells and cell walls of *M. tuberculosis* and do not seem to be secreted in culture fluids under normal culture conditions, although they are abundantly shed from dead mycobacterial cultures' velum (data not shown). Although they have been produced by chemical synthesis and present bioactivity similar to that of their non-nucleotide pyrophosphorylated counterparts, nucleotide conjugates UTP-X and TTP-X will not be discussed further in this review. The same organic moiety (X) is present in antigens TUBag 1, 3, 4 and was definitively identified 4 years later as a novel metabolite [39].

This compound (MW 262 Da, figure 1) is only bioactive as its native structure of pyrophosphorylated alde-

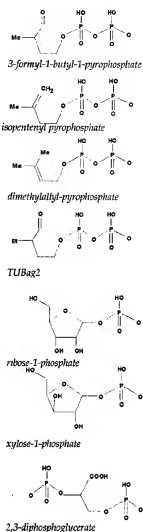


Figure 1. Different phosphorylated molecules activating G9D2 T cells.

hyde. The antigen identified by its molecular weight (MW) of 276 [40] corresponds to a structural analogue (MW+14) of 3-formyl-1-butyl-pyrophosphate and was also characterised as phosphoantigen corresponding to the non-nucleotide TUBag2 ligand [39]. These molecules have been found in *M. tuberculosis*, *M. bovis* and in *M. bovis* BCG, but they are also present in several mycobacteria from the non-tuberculous group such as *M. smegmatis*, *M. avium*, *M. phlei*, *M. fortuitum*, *M. chelonae*, *M. vaccae*, *M. scrofulaceum*, *M. marinum* and *M. kansasii* [41, 42].

Similar studies by C. Morita and colleagues [40, 43, 44], focused on *M. smegmatis* and *M. fortuitum*, had led to the identification of isopentenyl pyrophosphate (MW 246, see figure 1 with its bioactive dimethylallyl isomer) and to the detection of another bioactive compound of MW 276, which most presumably corresponds to the

above-mentioned TUBag2 molecule (for a possible structure, see figure 1). These isoprenoids can be isolated from culture media, as non-tuberculosis species seem to actively secrete such compounds in the surrounding media. Thus almost all mycobacteria species should be regarded as potentially or formally prone to phosphoantigen production. Parallel analysis from De Libero's group had indicated that besides 3-formyl-1-butyl pyrophosphate, characterised by its MW = 262 several naturally occurring phosphorylated metabolites such as ribose-1-phosphate, xylose-1-phosphate, 2,3-diphosphoglycerate and glycerol-3-phosphate activate the same G9D2 cells [45, 46].

However, there is so far no evidence for a role of these metabolites as mediators of phosphoantigenic activation by mycobacteria or their extracts. Other bioactive molecules with related structures, which were partially characterised or defined from studies using chemical synthesis and natural extracts are reviewed below.

2.2. Other natural phosphoantigens

The first biochemical evidence for non-mycobacterial phosphoantigens was based on the isolation of two G9D2 cell-stimulating compounds in *Plasmodium falciparum* (MALag1 and 2), separated by HPLC, with bioactivity sensitive to treatment by phosphatases but not by proteases [47, 48]. Other eukaryote parasites such as *Toxoplasma gondii* [49] and *Leishmania* spp. may produce structurally related ligands, but direct evidence for their presence is still lacking [35, 50–60]. In plants, phosphoantigens have been evidenced as well, as in *Viscum album* (mistletoe) water-soluble extracts [61, 62]. In prokaryotes, Gram-positive bacteria with related stimuli are: *Micrococcus luteus*, *Streptomyces noursei*, *Streptomyces griseus* [63], streptococci of group A, *Listeria monocytogenes* [64, 65], *Enterococcus faecalis*, *Corynebacterium diphtheriae* [66] and *Corynebacterium ammoniagenes* [63]. Similarly, the G9D2 cell-stimulating material from the Gram-negative bacterium *Francisella tularensis* was characterised as comprising at least two phosphoantigens [67]. More recent analysis has revealed its close structural relatedness to the mycobacterial 3-formyl-1-butyl pyrophosphate-containing molecules TUBag1 and TUBag3 (Kroca M., unpublished data). Other Gram-negative bacteria with related phenotype, although the underlying cytoplasmic ligands were not formally isolated, are *Pseudomonas aeruginosa* [68, 69], *Escherichia coli* [70], *Agrobacterium tumefaciens* [63], *Xanthomonas maltophilia* [66] and *Yersinia* spp. [70–74]. Note, however, that this list will soon be outdated, as these stimulating ligands are widespread.

2.3. Metabolic production of natural phosphoantigens

Until recently, the biosynthesis of isoprenoid was thought to proceed in nearly all living cells through a mevalonate pathway involving the key enzyme 3-hydroxy-3-methylglutarylcoenzyme A reductase (HMGR, EC1.1.1.34). On the other hand, the metabolic source of 3-formyl-1-butyl pyrophosphate has not been formally identified yet. However, recent developments in the biosynthesis of isoprenoid-containing compounds in bacteria, algae and plants have led to the discovery of a distinct

metabolic pathway which conceivably could account for the natural production of pyrophosphate-containing antigens. Initial observations in this direction were multiple. On the one hand, the gene for HMGR is absent from the mycobacterial genome [3], and a structural difference was demonstrated between isoprenoid metabolites and mycobacterial phosphoantigens [38, 39, 75]; on the other hand, several bacterial extracts with high isopentenyl diphosphate (IPP) contents lack G9D2 T-cell stimulus [63]. Very convincingly, using bacterial cultures, a correlate was found between incorporation of metabolic precursors of the Rohmer's pathway and production of the G9D2 stimuli [63]. Furthermore, the five-carbon aldehyde structure of 3-formyl-1-butyl pyrophosphate corresponds to a pyrophosphorylated precursor of IPP with an intermediate oxidation level expected for one of the last (yet unidentified) steps of the mevalonate-independent pathway [39, 76–78]. Considering the involvement of G9D2 T cells in anti-infectious immunity and their stronger activation by microbial metabolites than by eukaryote isoprenoid homologues, it was suggested that G9D2 cells discriminate self from non-self by focusing their activation on selective metabolic routes [80].

3. Synthetic phosphoantigen agonists

So far, dozens of phosphorylated compounds have been chemically synthesised and assayed on G9D2 cells to define their structure-activity relationship (SAR). Different compounds induce almost the same G9D2 cell responses, but when triggered by different concentration levels. Based on SAR studies reported by Y. Tanaka's [40, 43, 44], G. De Libero's [45, 46, 79, 80, 81] and our [75, 82] groups, one may now distinguish three main categories of G9D2 cell-stimulating, phosphorylated ligands. These are, respectively, agonists with bioactivities in the 10^{-1} – 10^{-3} M concentration range, classified as weak agonists, medium agonists bioactive in the 10^{-4} – 10^{-6} M window and strong agonists bioactive in the 10^{-7} – 10^{-10} M range. For easier reading, a compilation of these compounds is given in table 1, with their activities (EC_{50}) expressed as reported and usually detected bioactive window (μ M); for comparison, only non-nucleotide conjugates were included.

4. Functionally related molecules

4.1. Phosphoantagonists

In the course of SAR studies focussing on the role of the pyrophosphate moiety of phosphoantigens, novel synthetic molecules were produced wherein the interphosphorus oxygen link was replaced by CF_2 (methylene-diphosphonates), CHF , CF_3 (respectively mono- and difluoromethylene diphosphonates) or by NH (imido-diphosphonates). As expected, these structural changes proved to deeply affect the rate of pyrophosphate hydrolysis in phosphoantigens by $\gamma\delta$ cells, which was reduced in the order $O > NH > CH_2 > CHF > CF_2$ [82]. Parallel titrations of the G9D2-stimulating properties of these mol-

Table I. Non-nucleotide phosphoantigens.

Compound	EC ₅₀ range (µM)	Reference
<i>Weak agonists</i>		
Ribose-1-phosphate	1 000–10 000	[44]
Sec-butyl-phosphate*	1 000–5 000	[42]
Phospholactic acid*	500–5 000	our unpublished result
2,3-Diphosphoglycerate	500–5 000	[44]
Xylose-1-phosphate	500–5 000	[44]
Glycerol-3-phosphate	500–5 000	[44]
Methyl-phosphate*	500–1 000	[42]
Propyl-phosphate*	500–1 000	[42]
Isopropyl-phosphate*	500–1 000	[42]
Phosphoglycolic acid	500–1 000	[42]
<i>Medium agonists</i>		
β-hydroxyethyl-phosphate*	100–500	[42]
Isopentenyl-phosphate	50–500	[41]
γ-butyrolactone-α-pyrophospho ester	50–500	our unpublished result
Allyl-phosphate	50–500	[41]
Crotyl-phosphate	50–500	[41]
Dimethylallyl-phosphate	50–500	[41]
2-Butanon-1-yl-pyrophosphate	20–100	[38]
3-Pentanon-1-yl-pyrophosphate	10–100	[38]
4-Pentanon-1-yl-pyrophosphate	10–100	[38]
2-Methyl-3-butanon-1-yl-pyrophosphate	10–100	[38]
3-Butanon-1-yl-pyrophosphate	20–80	[81]
α,γ-Dibromohydrin triphosphodiester	10–20	[81]
α,γ-Diiodohydrin triphosphodiester	5–15	[81]
2-Me-iodohydrin pyrophosphate	5–10	[81]
3-Methyl-3,4-butanediol-1-yl-pyrophosphate	1–10	[81]
Allyl-pyrophosphate	1–10	[41]
Crotyl-pyrophosphate	1–10	[41]
Dimethylallyl-pyrophosphate	1–10	[41]
Ethyl-pyrophosphate	1–10	[42]
Isopentenyl-pyrophosphate	1–10	[41]
Pamidronate	4–8	[82]
Atendronate	0.9	[83]
Ibandronate	1	[83]
<i>Strong agonists</i>		
Bromohydrin triphosphate	0.080–0.150	[81]
Iodohydrin triphosphate	0.050–0.100	[81]
3,4-Epoxy-3-methyl-1-butyl pyrophosphate	0.020–0.080	[81]
3-Formyl-1-butyl-pyrophosphate	0.005–0.010	[38]
Chlorohydrin pyrophosphate	0.050–0.100	[81]
Bromohydrin pyrophosphate	0.005–0.020	[81]
Iodohydrin pyrophosphate	0.0005–0.003	[81]

*Activities estimated with crude chemical synthesis mixtures.

ecules revealed their concurrent switch towards loss of stimulatory properties. More interestingly, these diphosphonates behave as specific antagonists of activation by their pyrophosphate-containing counterparts. As usually reported for antagonist ligands, a 1 000-fold molar excess of the antagonist is necessary to achieve complete inhibition of γδ T-cell activation by phosphoantigens (using either cytotoxicity or TNF release readouts). However, as occurs with synthetic phosphoantigens, the presence of a chemically reactive group on the alkyl moiety enhances the inhibitory property of the phosphonate (Figure 2).

This antagonism acts at the level of initial activation events, is proper to phosphoantigen agonists, is fully revers-

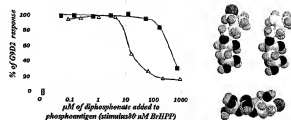


Figure 2. Antagonism of G9D2 cell response to phosphoantigen (BrHPP, bottom structure) by methylene diphosphonates (white triangles and upper left structure) BrHPPCH₂P or by Isopentenyl P-CH₂-P (black squares and upper right structure) P: yellow, C: white, O: red, H: blue, Br: green.

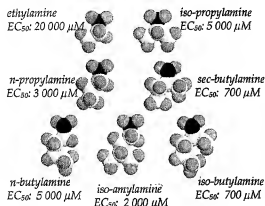


Figure 3. Different alkylamines activating G9D2 T cells and their bioactivity (EC_{50}).

ible by a law of mass action, and is not selective of a phosphoantigen ligand, while it does not inhibit lectin mitogen-induced activation. This underlines the importance of the phosphoantigen hydrolysis for triggering G9D2 activation ([82] and E. Espinosa et al., unpublished studies). Besides, these novel phosphonates might prove useful agents to selectively inhibit a deleterious $\gamma\delta$ T-cell activation by phosphoantigen overload.

4.2. Alkylamines

Another category of small non-peptide antigens harbouring an amino terminus was described recently [83]. These novel antigens (figure 3) constitute an interesting group for the following reasons: 1) they selectively activate the G9D2 cell subset, although they are not phosphorylated; 2) they are present in large amounts in microbial and plant extracts; 3) they may owe their bioactivity to a different SAR than phosphoantigens; 4) the immune response that they elicit represents another bridge between adaptive and innate immunity.

Butylamines are well known intermediates for pharmaceutical, dyeing and insecticidal agents; they have a low MW and are miscible with water. Furthermore, as these ammoniacal molecules can readily be obtained through chemical synthesis, they are prone to *in vivo* studies, which often demand large quantities of pure material.

However, one should mention potential limits to such studies due to their relative low bioactivity (in the millimolar concentration range for most of the bioactive compounds known so far [83]), taken together with their intrinsic toxicity. Alkylamines are potent irritants of skin, eyes and mucosae, causing severe blistering upon direct contact. They have oral LD_{50} measured in rats inferior to 7 mmol/kg or 500 mg/kg [84]. No γ -phosphorylated derivative of the above bioactive alkylamines has been found to be bioactive so far [83], although for several years, structurally related compounds have been developed pharmacologically and tested *in vivo* in human therapy (ABPs, see below). Hence, the role of bystander cells targeted by the toxicity of alkylamines cannot be ruled out yet as a potential source of direct stimulus for G9D2 T cells.

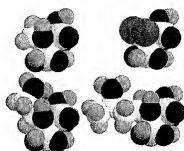


Figure 4. Therapeutic bisphosphonates used in bone resorption inhibition and in treatment of multiple myeloma. Top: G9D2-non-stimulating, non-amino molecules: etidronate (left), clodronate (right). Bottom: G9D2-stimulating aminobisphosphonates: pamidronate (left, EC_{50} : 4 μ M) and ibandronate (right, EC_{50} : 1 μ M).

4.3. Aminobisphosphonates (ABPs)

For their structural resemblance to pyrophosphate (figure 4), bisphosphonates (BPs) have been pharmacologically developed [85] and are now currently used as therapeutic agents inhibiting osteoclastic bone resorption [86]. Whereas initially, BPs were based on a methylene diphosphonate frame, further BP generations aiming at a higher resistance to phosphatases comprised halogenated species (e.g., clodronate) and alkyl or hydroxyl substitution of the central methylene unit (e.g., etidronate).

Novel BPs comprise an amino terminus at the straight chain or ramified alkyl segment (ABP): pamidronate, ibandronate, alendronate (figure 4). Therefore, these ABPs represent therapeutic molecules which share homologies with both phosphoantigens and the bioactive alkylamines mentioned above. Interestingly enough, ABP antiosteoclastic activity arises from inhibition of matrix metalloproteinases [87], alkaline phosphatase, pyrophosphatase [88] and of the mevalonate pathway farnesylpyrophosphate synthase [89].

An acute-phase reaction was classically known as side effect in some patients receiving their first ABP therapy [90]. A recent study has documented a strong expansion *in vivo* of G9D2 cells in such patients, whereas it did not occur with non-amino BPs [91]. Further *in vitro* analysis of this effect confirmed the relationship of such clinical outcomes to the direct G9D2-stimulation by ABPs, but not by non-amino-BPs [92]. These novel findings considerably enrich current knowledge on potential applications of human $\gamma\delta$ cell immunomanipulation. By drawing highly valuable observations from humans treated in controlled conditions with G9D2-specific agonist ligands, the future development of molecules and approaches will profit anticancer immunotherapy, for example, in multiple myeloma [93, 94].

5. Molecular basis of phosphoantigen recognition

5.1. A TCR-mediated recognition of phosphoantigen ligands

Current concepts about non-peptide antigen recognition by human $\gamma\delta$ T cells state that this reactivity is mediated by the TCR G9D2 [95, 96]. The rationale for this orthodox view relies upon analytical studies using clone collections which demonstrate the strict correlate of reactivity to phosphoantigens with cell surface expression of a G9D2-encoded TCR [31, 97–100]. Indeed, specific inhibition of this stimulation was reached when mAbs directed against G9D2 TCR were added to phosphoantigens in the reactive T-cell cultures [37, 40, 43], while unrelated mAbs were without effect. Definitive evidence was the transfection of the TCR G9 and D2 genes in a TCR⁺ T-cell line which transferred the reactivity to phosphoantigens [44, 101]. These observations suggested that germline residues specific to V gamma 9, V delta 2 and J gamma P elements directly contribute to recognition of phosphoantigens [100]. In addition, alteration of the genuine TCR gamma junctional regions (N and J) in a reactive G9D2 cell clone also abrogate this reactivity, emphasising the role of the γ -CDR3 loop and its adjacent residues [102].

5.2. Rapid chemical processing of phosphoantigen ligands by $\gamma\delta$ cells

Despite these convincing lines of evidence for the G9D2 TCR as receptor for phosphoantigens, this scene may yet turn out to be incomplete. Although experiments that fail are rarely published, other attempts to obtain activation of functional G9D2 TCR-transfected cells using phosphoantigens, however, repeatedly failed (our unpublished observations).

Furthermore, recent data have suggested that phosphoantigen-driven stimulation of $\gamma\delta$ cells may involve an unusual mode of ligand recognition. This comprises not only topological fit to the receptor but also a chemically based step of phosphoantigen degradation. This second step consists of pyrophosphate dephosphorylation and alteration of the organic segment [82]. While a mere catalytic activity of the G9D2 TCR is now excluded from this process, the contribution of a non-TCR component at the $\gamma\delta$ cell surface appears to constitute a key additional step to the TCR-mediated phosphoantigen binding (E. Espinosa, unpublished data).

On these grounds, chemically reactive groups of putative ligands strengthen the properties of phosphoantigens, either stimulatory or inhibitory, making these latter either $\gamma\delta$ cell agonists or antagonists [46, 82]. Molecules with topological resemblance to phosphoantigen agonists (e.g., BrHPP, figure 5) but resistant to pyrophosphate hydrolysis by $\gamma\delta$ cells (e.g., BrHP-NH-P; BrHP-CH₂-P or BrHP-CF₃-P, figure 5) specifically but reversibly inhibit the activation by phosphoantigens [82]. Thus agonist/antagonist molecules may look topologically related, although they are differently susceptible to dephosphorylation (figure 6).

In complete culture medium at 37 °C, this degradative recognition operates at a high rate on the outer cell surface, leading to a ligand consumption rate estimated at

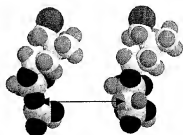


Figure 5. The strong agonist BrHPP (left) and its structurally related strong antagonist BrHP-CH₂-P (right). Same S enantiomers are shown, arrows indicate the interphosphorus-bridging atom for comparison.

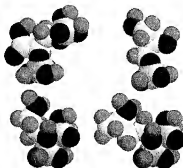


Figure 6. Topologically related phosphoantigens. A weak agonist/antagonist 2,3-DPG (upper left) and its related weak agonist 3P-glycerol (upper right). Below: pamidronate (left) and ibandronate (right), two aminobisphosphonates of the medium agonist category (deep blue: N and same colours as above).

about 1 000 BrHPP molecules s⁻¹ per $\gamma\delta$ cell, whereas it is slower with less stimulatory ligands (~ 850 IPP molecules, E. Espinosa unpublished data) under the same conditions.

Future studies will focus on several unresolved issues concerning $\gamma\delta$ cell stimulation by non-peptidic antigens: novel molecules with potentiated properties of either agonist or antagonist type, and identification of the molecular partners of the G9D2 partners involved in this unusual mode of antigen recognition.

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